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*Published in:*
Proceedings of the National Academy of Sciences

*DOI:*
10.1073/pnas.88.6.2471



*Publication date:*
1991

*Proc. Natl. Acad. Sci. USA*
Vol. 88, pp. 2471–2475, March 1991
Biochemistry

# Proton transfer is rate-limiting for translocation of precursor proteins by the *Escherichia coli* translocase

(secretion/Sec proteins/proOmpA/protonmotive force)

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*Communicated by Paul D. Boyer, December 6, 1990*

**ABSTRACT** **The protonmotive force stimulates translocation *in vivo*, in crude *in vitro* reactions, and in a purified, reconstituted reaction. Translocation activity is a function of the pH at the inner face of the membrane. Both the transmembrane pH gradient and the transmembrane electrical potential stimulate translocation. A late-stage translocation intermediate of the proOmpA preprotein completes its translocation in the absence of ATP when a protonmotive force is imposed. This completion of translocation is retarded by a factor of >3 in deuterium oxide relative to water, demonstrating that translocation involves proton-transfer reactions in rate-limiting steps.**

Translocation of precursor proteins across the inner membrane of *Escherichia coli* is catalyzed by the preprotein translocase (1) and requires two energy sources, ATP and the protonmotive force (pmf). Prior to translocation, precursor proteins may form transient complexes with the SecB protein (2–5). SecA is a peripheral membrane protein (6) that binds the SecB/preprotein complex (7). SecA is an ATPase (8) and requires acidic phospholipids (ref. 9; J. Hendrick and W.W., unpublished data) and the SecY/E protein (1, 7) for membrane binding and activity. ATP hydrolysis promotes the transient dissociation of the SecA/precursor protein complex to allow unidirectional translocation in distinct steps (10).

The SecY and SecE proteins have been purified as a complex of integral membrane polypeptides that function together with the SecA protein as a translocase (1). Precursor proteins might translocate through the lipid phase *per se* (11), along the surface of the SecY/E protein, or through its center.

*In vivo* translocation requires a pmf (12–17). *In vitro* experiments have shown that the requirement for ATP is absolute, and the pmf stimulatory (1, 18–20). That the pmf is only stimulatory *in vitro*, whereas there is a clear requirement for it *in vivo*, led to the suggestion that the role of the pmf is indirect (18). However, the recent observation that the pmf is strongly stimulatory in a reconstituted reaction using all purified components argues against an indirect role of the pmf (1, 21).

Using the purified components proOmpA, SecB, SecA, and the SecY/E protein, we now show that both a transmembrane pH gradient (ΔpH) and a transmembrane electrical potential ($\Delta\psi$) stimulate the rate of translocation, as has been shown *in vivo* (17) and in crude cell-free extracts (22). Conditions that separate the requirement for ATP hydrolysis and the pmf reveal a critical proton-transfer reaction. These results support a direct role of the pmf in translocation.

## MATERIALS AND METHODS

**Materials and Bacterial Strains.** Inverted inner membrane vesicles (23) were from *E. coli* KM9 (24). SecA protein (24) was from strain BL21(λDE3)/pT7-secA (25). SecB was purified (3) from BL21(λDE3)/pJW25 (2). SecY/E protein (1) was from strain UT5600 (26). ProOmpA was prepared as described (27, 28) and dissolved in 6 M urea/1 mM dithiothreitol/20 mM Tris·HCl, pH 8.0. [$^{35}$S]ProOmpA was synthesized *in vitro* and purified (29). Deuterium oxide ($D_2O$; >99% pure) was from Aldrich. Creatine kinase and proteinase K were from Boehringer Mannheim. Oxonol VI [bis(3-propyl-5-oxoisoxazol-4-yl)pentamethine oxonol], pyranine (8-hydroxy-1,3,6-pyrenetrisulfonate), and 9-amino-6-chloro-2-methoxyacridine were from Molecular Probes.

**Translocation Assay.** Translocation of [$^{35}$S]proOmpA into membrane vesicles and proteoliposomes was assayed by its inaccessibility to added proteinase K (24). Initial rates were estimated after 5 min. Samples were treated with proteinase K (1 mg/ml, 15 min, 0°C) and analyzed as described (28, 30).

**Other Methods.** SecY/E protein was reconstituted into liposomes composed of *E. coli* phospholipid (1). Translocation ATPase was assayed according to Lill *et al.* (8). ΔpH was measured with the fluorescent dye 9-amino-6-chloro-2-methoxyacridine (31). Excitation and emission values were measured at 409 and 474 nm using slit widths of 10 nm. $\Delta\psi$, inside positive, was determined with oxonol VI (32). Excitation and emission were at 599 and 634 nm using slit widths of 5 nm. Protein (33) and lipid phosphorus (34) were determined as described. To study deuterium solvent effects, solutions were prepared in $D_2O$ (>99%) and adjusted with concentrated HCl or KOH such that the pH measured with a hydrogen-ion-selective glass electrode was 0.4 unit more acid than the pD desired (i.e., pD = pH + 0.4) (35).

## RESULTS

**Rate of Translocation Is Stimulated by the pmf.** Translocation of proOmpA into inner membrane vesicles requires ATP and is stimulated by the pmf (19). In this study, we used membranes from *E. coli* KM9 (31), which lack the $H^+$-translocating ATPase. These vesicles cannot generate a pmf by ATP hydrolysis, though they generate a pmf when supplied with NADH. In the presence of SecB and limiting amounts of membranes, proOmpA translocation was linear for 15 min (data not shown) and increased with the ATP concentration (Fig. 1, ■). The translocation rate was enhanced by NADH (□), while the apparent $K_m$ for ATP remained essentially the same. An excess of purified SecA protein, 40 μg/ml, was used in these experiments. Although efficient translocation of proOmpA was readily observed in the absence of a pmf, the stimulatory effect of the pmf on the

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Abbreviations: ATP[β,γ-NH], adenosine 5′-[β,γ-imido]triphosphate; BSA, bovine serum albumin; $D_2O$, deuterium oxide; pmf, protonmotive force; ΔpH, transmembrane pH gradient; $\Delta\psi$, transmembrane electrical potential.

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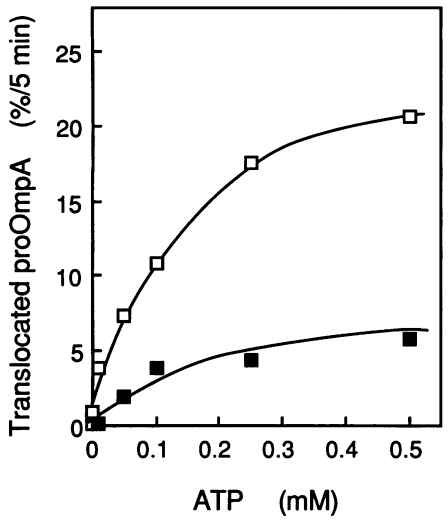


FIG. 1. The pmf enhances the rate of translocation. The initial rate of proOmpA translocation by *E. coli* KM9 membrane vesicles was assayed at 1–500 μM MgATP in the absence (■) or presence (□) of 5 mM NADH. Reactions were performed in buffer A (50 mM Tris·HCl, pH 8.0/30 mM KCl/30 mM $NH_4Cl$/1 mM dithiothreitol) with $MgSO_4$ (1 mM), fatty acid-free bovine serum albumin (BSA, 0.5 mg/ml), SecA (40 μg/ml), SecB (5 μg/ml), MgATP, inner membranes (30 μg of protein per ml), creatine phosphate (10 mM), creatine kinase (10 μg/ml), and [$^{35}$S]proOmpA (70,000 cpm per sample).

rate of proOmpA translocation was still seen in the presence of a high concentration of SecA (Fig. 1). Half-maximal rates of translocation of proOmpA required 2–3 μg of SecA protein per ml in either the presence or the absence of a pmf (data not shown). Thus ATP is required for translocation whereas the pmf facilitates translocation by enhancing its rate.

**High-Affinity Binding of ProOmpA to the Translocase Is Independent of pmf.** SecA-dependent binding of a low concentration of proOmpA (5 nM) to membrane vesicles was measured in the absence and presence of NADH. SecB (5 μg/ml) was included to suppress nonspecific interactions with the membrane surface (7). In the presence of SecA (20 μg/ml), the ratio of proOmpA that specifically bound to membrane vesicles to free proOmpA was 0.48 ± 0.03 ($n$ = 3) at 20°C. Binding was not significantly increased by NADH (bound/free = 0.55 ± 0.03) or by NADH plus the ionophores valinomycin (0.5 μM) and nigericin (0.1 μM) (bound/free = 0.57 ± 0.05). Similar results were obtained at 0°C (data not shown). Thus, pmf is not required for the initial binding of proOmpA.

**Translocation and Translocation ATPase Activities Depend on the Cytosolic pH.** To separate the roles of the individual components of the pmf from effects of pH, we studied the pH profiles of translocation ATPase and translocation. ATP hydrolysis by the SecA protein is stimulated by proOmpA (8, 27) and requires the interaction of SecA with acidic phospholipids (9) and the SecY/E protein (1, 7). Translocation ATPase activity increased with pH (Fig. 2*A*) with an apparent pK ($pK_a$) of 6.7. A Hill plot (Fig. 2*A Inset*) suggests that inactivation results from protonation of a single site or a set of identical sites. The initial rate of proOmpA translocation (Fig. 3, lane 2) increased with pH and closely correlated with translocation ATPase (Fig. 2*A*). To distinguish between a

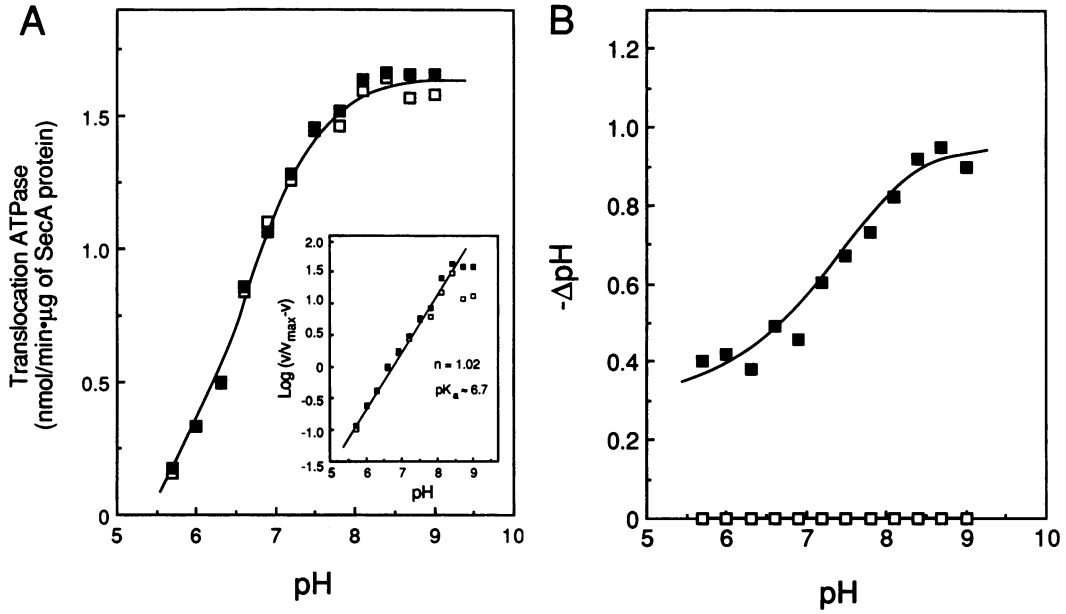


FIG. 2. Translocation ATPase is dependent on the cytosolic pH. (*A*) Translocation ATPase assays were in buffer B (30 mM potassium Mes/30 mM potassium Hepes/30 mM potassium Tricine/50 mM KCl/1 mM dithiothreitol) of the indicated pH containing $MgSO_4$ (5 mM) and BSA (0.5 mg/ml). Reaction mixtures contained inner membranes (20 μg/ml), ATP (4 mM), NADH (5 mM), SecA (20 μg/ml), SecB (80 μg/ml), and valinomycin (1 μM). ProOmpA was diluted 50-fold from 6 M urea to a concentration of 40 μg/ml. Assays were performed in the absence (■) or presence (□) of nigericin (0.1 μM) to vary the intravesicular pH. Samples (50 μl) were assayed every 10 min for inorganic phosphate (9). Translocation ATPase activity is the difference between phosphate release in the presence and absence of proOmpA. (*Inset*) Hill-plot analysis of translocation ATPase in the absence (■) or presence (□) of nigericin. (*B*) ΔpH (inside acid) was measured with pyranine. Inner membranes (10 mg/ml) were suspended in buffer B containing 200 μM pyranine and then were rapidly frozen in liquid nitrogen. Frozen suspensions of inner membranes were slowly thawed on ice, then dispersed by brief sonication (8 sec) in a bath sonicator. Untrapped pyranine was removed by gel filtration (Sephadex G-25 medium, Pharmacia; 1 × 25 cm). For fluorescence measurements, inner membranes (≈0.4 mg of protein) were diluted into buffer B (1.5-ml final volume) of the indicated pH containing $MgSO_4$ (5 mM), valinomycin (1 μM), SecA (10 μg/ml), proOmpA (40 μg/ml), ATP (4 mM), and BSA (0.5 mg/ml). The intravesicular pH was allowed to equilibrate with the medium pH, then energization was initiated by the addition of 5 mM NADH. Pyranine fluorescence intensity levels were determined in the absence (■) or presence (□) of nigericin (0.1 μM). Excitation and emission values were measured at 460 and 510 nm using slit widths of 5 nm (36). The intravesicular pyranine intensity as a function of pH was calibrated by adjusting the medium pH in the presence of 0.1 μM nigericin (37).

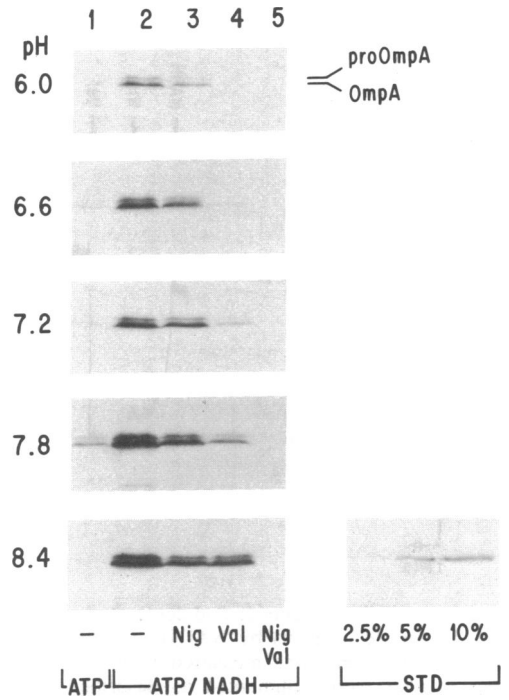


FIG. 3. Optimal translocation requires the total pmf. The initial rate of proOmpA translocation into membrane vesicles was assayed in the presence of ATP (lanes 1–5) and NADH (lanes 2–5) in the presence of nigericin (Nig; lanes 3 and 5) or valinomycin (Val; lanes 4 and 5). Translocation reactions were in buffer B of the indicated pH containing 5 mM $MgSO_4$. Reaction mixtures contained inner membranes (30 μg/ml), SecA (40 μg/ml), SecB (5 μg/ml), [$^{35}$S]proOmpA (70,000 cpm per sample), BSA (0.5 mg/ml), and, where indicated, ATP (2 mM), NADH (5 mM), nigericin (0.1 μM), and valinomycin (1 μM). Standards (STD) of the indicated percent of total input proOmpA are shown.

cytosolic and a periplasmic pH effect on the activity of the translocase, translocation ATPase was measured in the presence of NADH under conditions where the intravesicular pH—i.e., the pH on the periplasmic side—was controlled by the ionophore nigericin. Nigericin catalyzes the electroneutral exchange between $H^+$ and $K^+$, thereby dissipating ΔpH at the expense of a $K^+$ gradient. The $K^+$ ionophore valinomycin (0.5 μM) was included to collapse $\Delta\psi$. The pH on the periplasmic side was quantified with the fluorescent pH indicator pyranine (36). In the presence of both ionophores, $pH_{in}$ equaled $pH_{out}$ (Fig. 2*B*, □). In the absence of nigericin, $pH_{in}$ was more acidic than $pH_{out}$ and varied with the pH of the medium according to $pH_{in} = pH_{out} + \Delta pH$. The pH dependency of translocation ATPase (Fig. 2*A*) was identical when assayed in the presence of nigericin (□) or in its absence (■). These results suggest that the activity of the translocase is not modulated by the periplasmic pH but rather is affected by the cytosolic pH.

**Optimal Translocation Requires a pmf.** NADH accelerates translocation over the wide pH range 5.7–9.0 (Fig. 3, lanes 1 and 2). The effects of dissipation of ΔpH and $\Delta\psi$ by nigericin and valinomycin were analyzed at different pH. Valinomycin strongly reduced the rate of proOmpA translocation (lanes 2 vs. 4). Nigericin had only a moderate effect (lanes 3). Translocation was completely suppressed when both ionophores were present (lanes 5). The rate of proOmpA translocation was reduced to a level below that observed with ATP alone, suggesting that ATP can generate a small pmf by means other than the $F_1F_0$-ATP synthase. These results indicate that the effect of NADH on the translocation of proOmpA involves both components of the pmf.

The low proton permeability of proteoliposomes allows the generation of a stable pmf by diffusion gradients of ions and weak acids. The purified SecY/E protein was reconstituted into proteoliposomes in $Na^+$ salts. These proteoliposomes were diluted into a medium containing KOAc and valinomycin to create both a $\Delta\psi$ (inside positive) and a ΔpH (inside acid). Optimal rates of translocation of proOmpA into these proteoliposomes required ATP (1) and was stimulated by the combined imposition of ΔpH and $\Delta\psi$ (Fig. 4). Little stimulation was seen with either a $\Delta\psi$ or a ΔpH alone. Thus both the ΔpH and the $\Delta\psi$ are required for optimal translocation of proOmpA.

**pmf-Dependent Translocation Exhibits a Major Deuterium Solvent Isotope Effect.** A translocation intermediate of proOmpA accumulates transiently in the absence of a pmf (38). In the absence of ATP, further translocation of this intermediate proceeds to completion when a pmf is imposed (38, 39). Many enzymatic reactions involving proton transfer in the rate-limiting step proceed at a slower rate in the presence of deuterium relative to protium (35, 40). To test whether deprotonation or protonation may be rate-determining for pmf-dependent proOmpA translocation,

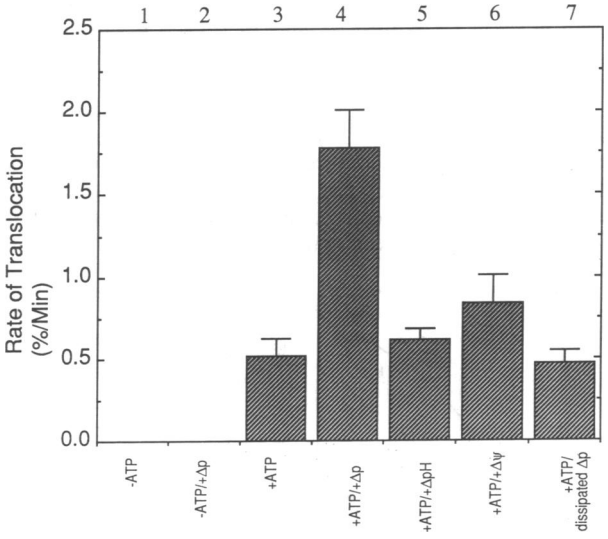


FIG. 4. Rate of translocation is stimulated by a pmf. Translocation of proOmpA by reconstituted SecY/E proteoliposomes was assayed in the presence of ATP (lanes 3–7) or in its absence (lanes 1 and 2) upon the imposition of both $\Delta\psi$ and ΔpH (Δp; lanes 2, 4, and 7), $\Delta\psi$ (lane 6), ΔpH (lane 5), or no gradient (lanes 1 and 3). In lane 7, the imposed pmf was allowed to collapse for 10 min prior to the addition of ATP, SecA and [$^{35}$S]proOmpA. SecY/E proteoliposomes were formed by detergent dilution into 50 mM Tris·HCl, pH 8.0/50 mM NaCl/1 mM dithiothreitol. Proteoliposomes were precipitated by $Ca^{2+}$, collected by centrifugation (41,000 × *g*, 45 min, 4°C), and resuspended in buffer with 2.5 mM EGTA (1). Valinomycin (2 nmol per mg of phospholipid) was added and proteoliposomes were collected by centrifugation [Beckman Airfuge, 30 psi (1 psi = 6.89 kPa), 1 hr, 4°C]. Pellets were resuspended in 50 mM Tris·HCl, pH 8.0/50 mM NaCl/1 mM dithiothreitol to a phospholipid concentration of ≈20 mg/ml and bath-sonicated for 8 sec. Translocation mixtures (300 μl) contained [$^{35}$S]proOmpA (400,000 cpm per reaction), Tris·HCl (50 mM, pH 8.0), dithiothreitol (1 mM), $MgSO_4$ (5 mM), SecA (20 μg/ml), SecB protein (5 μg/ml), BSA (0.5 mg/ml), valinomycin (1 μM), and a salt (50 mM) chosen according to the desired pmf: NaCl (no gradient), KCl ($\Delta\psi$, inside positive), NaOAc (ΔpH, inside acid), or KOAc (pmf, inside positive and acid). Where indicated, ATP was added to a final concentration of 2 mM. Reaction mixtures were preincubated at 40°C for 50 sec prior to the addition of proteoliposomes (4 μl). Translocation was started after 10 sec by the addition of [$^{35}$S]proOmpA. Samples (45 μl) were removed after 0.5, 1, 1.5, 2, 3, and 4 min, chilled on ice, and assayed for translocation. The initial rate of proOmpA translocation was measured. Each result is the mean of three assays with the indicated SE.

translocation was completed without ATP in $H_2O$ and $D_2O$ at an equivalent pH and pD of 8.4. By using the pmf alone to complete translocation, we studied the effect of deuterium without interference from reactions that involve ATP hydrolysis. The translocation intermediate of proOmpA accumulated when translocation was performed at a low ATP concentration (10 $\mu$M) for a limited period of time (10). Unbound proOmpA and residual ATP were removed by sedimenting the vesicles through 0.2 M sucrose. Vesicles were resuspended in buffer made in either $H_2O$ or $D_2O$, collected by centrifugation, and suspended in fresh buffer. Completion of translocation was initiated by the addition of NADH. Adenosine 5'-[$\beta,\gamma$-imido]triphosphate (ATP[$\beta,\gamma$-NH], 10 mM) was used to prevent hydrolysis of remaining traces of ATP (<1 nM). In the presence of a pmf, the intermediate slowly completes translocation. The rate of translocation was significantly reduced in $D_2O$ relative to $H_2O$ (Fig. 5*A*), while, at longer times, translocation proceeded to similar extents. The effect of $D_2O$ was not due to altered viscosity (data not shown). Dissipation of the pmf by valinomycin and nigericin blocked the chase (Fig. 5*A*). Deuterium had no effect on the NADH-driven generation of a $\Delta\psi$, monitored by the fluorescence quenching of oxonol VI (Fig. 6). The fluorescence quantum yield of oxonol VI was increased only slightly in $D_2O$ (Fig. 6). These results indicate that translocation is limited by proton transfer.

Since deuterium shifts the $pK_a$ values of acids to more alkaline values by 0.3–0.7 pH unit (35, 40), the observed effect of $D_2O$ might have represented a $pK_a$ effect rather than a true kinetic isotope effect. To differentiate between these two possibilities, the relative rates of the chase in $H_2O$ and $D_2O$ were compared over an extended range of pH (pD)

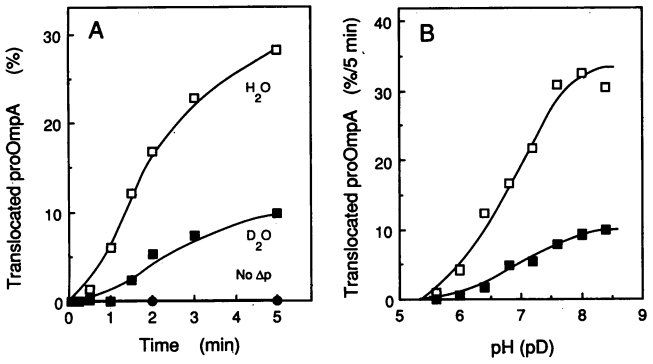


FIG. 5. pmf-dependent translocation exhibits a major deuterium solvent isotope effect. (*A*) The chase of intermediate to the fully translocated proOmpA was assayed in aqueous (□) or deuterated (■) buffer upon addition of NADH in the absence (■, □) or presence (●, ○) of nigericin and valinomycin (no $\Delta p$). $I_{26}$, a translocation intermediate of proOmpA with 26 kDa of translocated polypeptide chain (10, 38), accumulated in the presence of 10 $\mu$M ATP (10). Translocation reactions were performed for 5 min at 40°C as described in the legend to Fig. 1. Vesicles with intermediate from 0.5 ml of the reaction mixture were sedimented through 4.2 ml of sucrose solution (buffer A, pH 8.0, containing 0.2 M sucrose) at 250,000 × *g* for 45 min at 4°C. Pellets were resuspended in either aqueous or deuterated buffer A (pH/pD 8.4), collected by centrifugation (250,000 × *g*, 30 min, 4°C), and suspended as a concentrated solution. Membranes were diluted 10-fold into buffer A (pH/pD 8.4) containing 2.5 mM $MgSO_4$ (2.5 mM), ATP [$\beta,\gamma$-NH] (10 mM), and BSA (0.2 mg/ml). Nigericin and valinomycin were at 0.1 and 1 $\mu$M, respectively. Chase was initiated by 5 mM NADH. (*B*) Completion of translocation was assayed as a function of pH (pD) in aqueous (□) and deuterated (■) buffer in the presence of NADH. The intermediate of proOmpA was accumulated and isolated as described above. Membrane vesicles were diluted 10-fold into buffer B of the indicated pH (or pD) containing $MgSO_4$ (2.5 mM), ATP[$\beta,\gamma$-NH] (10 mM), and BSA (0.2 mg/ml). The reaction was started by the addition of 5 mM NADH, and fully translocated proOmpA was determined after 5 min at 40°C.

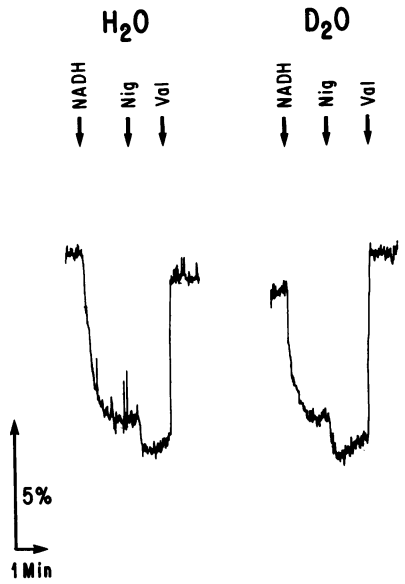


FIG. 6. $\Delta\psi$ is similar in aqueous and deuterated solvent. Generation of a $\Delta\psi$ (inside positive) by NADH in aqueous ($H_2O$) and deuterated ($D_2O$) solvent was measured with oxonol VI. Inner membrane vesicles were sedimented as described for Fig. 5*A*, resuspended in either aqueous or deuterated buffer A (pH/pD 8.4), and collected by centrifugation (250,000 × *g*, 30 min, 4°C). For fluorescence measurements, membrane vesicles (0.1 mg/ml) were diluted into 2 ml of aqueous or deuterated buffer A (pH/pD 8.4) containing $MgSO_4$ (2.5 mM) and BSA (0.5 mg/ml). Oxonol VI (2 $\mu$M) was added from a 1 mM stock solution in dimethyl sulfoxide. The reaction was initiated by addition of 5 mM NADH. Nigericin (Nig) and valinomycin (Val) were added to final concentrations of 0.1 and 1 $\mu$M, respectively.

values (Fig. 5*B*). The level of translocation determined after a 5-min incubation increased sigmoidally with pH, with a $pK_a$ of 6.8. The pD profile for translocation was also sigmoidal, with a $pK_a$ of 7.0. The deuterium solvent isotope effect remained essentially constant over the entire pH (pD) range (Fig. 5*B*). These results provide further evidence that translocation involves a critical proton-transfer reaction in rate-determining steps.

## DISCUSSION

SecA couples the hydrolysis of ATP to translocation (8). However, our kinetic studies indicate that the pmf requirement cannot be replaced by excess SecA and ATP (Fig. 1). Recent studies (10) define a catalytic cycle of translocation and show that pmf and ATP act at different parts of that cycle. A limited translocation of SecA-bound intermediate is driven by the energy of ATP binding, while ATP hydrolysis drives the release of the intermediate from SecA. The pmf then both prevents a reversal of translocation by this released intermediate and drives a rapid and efficient forward translocation reaction (10). Thus, though the role of the pmf is not yet defined, it is different from that of ATP hydrolysis.

Translocation reactions performed with either membranes (19) or SecY/E proteoliposomes (1) indicate that the rate of translocation is stimulated by the pmf. Both $\Delta\psi$ and $\Delta$pH stimulate translocation (Fig. 3). Translocation ATPase and translocation activities are modulated by the pH on the cytosolic face of the membrane (Fig. 2). However, for the well-buffered *in vitro* reaction, secondary effects arising from changes in pH can be excluded. We therefore propose that $\Delta$pH primarily acts as an energy source for translocation. Our studies indicate that translocation is limited by a critical proton transfer reaction. Moreover, the kinetic solvent isotope effect suggests that catalytic protons take part in the

intrinsic mechanism of translocation. With simple acid–base reactions, or ordinary hydrogen bonds that stabilize the three-dimensional structure of proteins, net isotope effects are usually quite close to unity (40, 41). Our results are therefore most easily understood if vectorial protons are involved in the translocation process. It seems unlikely that proOmpA would, during translocation, undergo multiple rounds of dissociation from SecY/E and reassociation. We therefore believe it unlikely that the deuterium solvent isotope effect (Fig. 5) is due to an association/dissociation reaction (35).

The role of the pmf in promoting translocation may reflect its direct effects on the precursor proteins, its ability to "energize" SecY/E to promote transport during its catalytic cycle, or both. Insofar as the pmf acts directly on the preproteins during translocation, the roles of $\Delta\psi$ and $\Delta$pH may be distinct. Since translocation is readily reversible (10), $\Delta$pH may drive net translocation by promoting the deprotonation of basic residues prior to translocation and their protonation afterwards. $\Delta\psi$ may have an electrophoretic effect on promoting the transfer of acidic regions bearing net charge across the membrane. These possibilities do not exclude a role of the pmf in energizing the SecY/E domain of translocase. However, the stimulation of translocation by the pmf is strikingly different for different precursors, each dependent on SecY/E (13, 22, 42). This suggests that the pmf does not promote translocation solely by energizing SecY/E to act as a proton/protein antiport (or hydroxyl–protein symport).

We thank Douglas Geissert and Marilyn Rice for expert technical assistance. This work was supported by a grant to A.J.M.D. from the Netherlands Organization for Scientific Research and by a grant to W.W. from the National Institute of General Medical Sciences.

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